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Intergeneric Conjugation between *Escherichia coli* and *Streptomyces* Species

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We have constructed *Escherichia coli-Streptomyces* shuttle plasmids which are capable of conjugal transfer from *E. coli* to *Streptomyces* spp. These plasmids contained the pBR322 and pIJ101 origins of replication and the RK2 (IncP) origin of transfer. The transfer of plasmid was specifically dependent the presence of a 760-base-pair, *cis*-acting, *oriT*-containing fragment and on RP4 (IncP) functions supplied in *trans*. Conditions of mating and selection of exconjugants were analyzed with *Streptomyces lividans* as recipient. Plasmid transfer to other *Streptomyces* species was also demonstrated.

It was long assumed that plasmids could not be naturally transferred between gram-negative and gram-positive bacteria. Recently Trieu-Cuot et al. (13, 14) dispelled this misconception when they demonstrated conjugation between gram-negative *Escherichia coli* and various gram-positive bacteria, including *Enterococcus faecalis*, *Streptococcus lactis*, *Streptococcus agalactiae*, *Bacillus thuringiensis*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The bifunctional vector they used (pAT187) contained the origins of replication of pBR322 and the broad-host-range streptococcal plasmid pAMβ1 (4). pAT187 contained an aminoglycoside phosphotransferase gene (*aphA3*) which was expressed in both gram-negative and gram-positive bacteria as a selectable marker. Transfer was dependent on the origin of transfer (*oriT*) of the IncP plasmid RK2 and the *tra* gene functions of RP4.

The work of Trieu-Cuot et al. is of interest with respect to the evolution of bacterial genes as well as from a technical point of view and may have special implications for another group of gram-positive organisms, the genus *Streptomyces*. *Streptomyces* spp. are differentiating, filamentous eubacteria which produce most antibiotics (1). The high G+C content and morphological properties of *Streptomyces* spp. indicate that these species are evolutionarily far removed from all of the bacteria mentioned above (16). DNA sequence comparisons have indicated that these species may have been the source of clinical antibiotic resistance genes (11, 15; J. E. Suárez, C. J. Villar, P. Arca, and C. Hardisson, Program Abstr. 4th ASM Conf. Genet. Mol. Biol. Ind. Microorg., abstr. no. 11, p. 17, 1988) and eucaryotic antibiotic production genes (9).

Applications of recombinant DNA technology in streptomycetes have increased rapidly in recent years (5). Nevertheless, genetic manipulations in these organisms have limitations compared with manipulations in *E. coli*. Streptomycetes grow slowly, and recombinant plasmids are sometimes unstable. Some basic techniques such as transposon mutagenesis and cosmid cloning are as yet poorly developed. Because of these limitations, *E. coli-Streptomyces* shuttle plasmids have been made which allow constructions in *E. coli* to be introduced into streptomycetes by transformation. The transformation protocol is tedious if one needs to transfer and test many constructions. In addition, while the procedure has been optimized for the genetically well-characterized strains *Streptomyces lividans* and *Streptomyces coelicolor*, it is not readily applicable to many other important antibiotic-producing species. Here we describe a shuttle plasmid which can be transferred from *E. coli* to *Streptomyces* spp. by simple conjugation.

Construction of conjugative *E. coli-Streptomyces* shuttle plasmids. Although pAT187 is transferable from *E. coli* to a variety of gram-positive organisms, we were unable to demonstrate stable replication in *S. lividans*. Attempts to introduce pAT187 to *S. lividans* by conjugation (from *E. coli*) or transformation were unsuccessful. It seems likely that pAT187 (pAMβ1) is unable to replicate in *S. lividans*. We therefore constructed a similar shuttle vector with the origin of replication of the streptomycete plasmid pIJ101 instead of pAMβ1. The constructions of the shuttle vector pPM801 and pPM803 are shown in Fig. 1. Both plasmids contain the pBR322 replicon and a 760-base-pair fragment containing *oriT* of RK2. pPM801 contains the entire pIJ101 replicon, which includes streptomycete spread and transfer functions (6). pPM803 was constructed from the streptomycete vector pIJ699 (7). pIJ699 is a pIJ101-derived vector which is defective for transfer in streptomycetes. To avoid an instability problem often encountered in *E. coli-Streptomyces* shuttle vectors, transcriptional terminator sequences between the two replicons were incorporated into pIJ699.

Intergeneric conjugation. Donor cultures of *E. coli* S17-1 carrying an integrated RP4 derivative (10) were prepared by growth overnight at 37°C in Luria broth (LB; 8) supplemented with neomycin (30 μg/ml). The bacteria were washed twice and resuspended in the same volume of LB. *S. lividans* spores were induced to germinate by heat shock (5), pelleted by centrifugation, and then resuspended in LB at a concentration of 10^8 cells per ml. Donors (0.1 ml; ca. 2×10^8 *E. coli* cells) and recipients (0.1 ml; ca. 10^7 *S. lividans* spores) were spread on LB (8) plates and grown overnight at 30°C. The plates were washed with LB and gently scraped with a pipette until the *E. coli* layer was removed and the mycelial *Streptomyces* cells could be seen still attached to the agar. Plates were then dried and covered with 1 ml of LB containing 100 μg of neomycin and 200 μg of nalidixic acid. The natural resistance of many streptomycetes to nalidixic acid was used to counterselect the sensitive *E. coli* donor. Exconjugants were counted 6 days later. Transfer from *E. coli* S17-1 containing pPM801 or pPM803 generated 2,800 or

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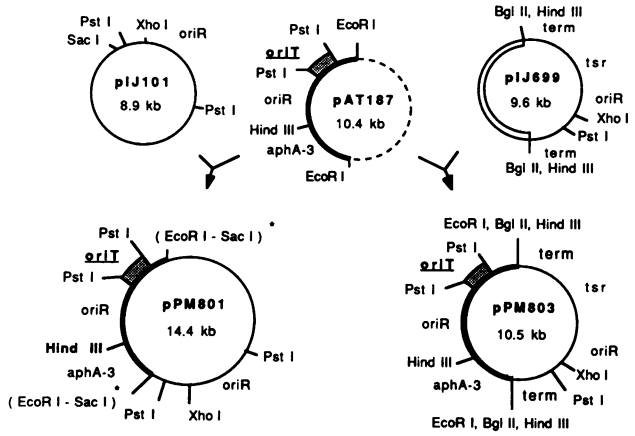

FIG. 1. Construction and restriction maps of plasmids pPM801 and pPM803. pPM801 was obtained by ligating the 5-kilobase (kb) *Eco*RI fragment of pAT187 to the *Sac*I-linearized pIJ101 by using a polylinker (described below) cleaved with *Eco*RI and *Sac*I. pPM803 was obtained by ligating the 5-kilobase *Eco*RI fragment of pAT187 to the 5-kilobase *Bgl*II fragment of pIJ699 (7) by using the same linker cleaved with *Bgl*II. ——, pIJ101 and derivatives; – – –, derived from pAMβ1; □, derived from p15A; ■, derived from pBR322; ▩, RK2 *oriT*; *, polylinker containing sites for *Eco*RI, *Bgl*II, *Mlu*I, *Bam*HI, and *Sac*I; term, bacteriophage fd transcriptional terminator; oriR, origin of replication; tsr, thiostrepton resistance gene; aphA-3, neomycin resistance gene. The unique *Hin*dIII site available for cloning in pPM801 is indicated by boldface type.

1,200 exconjugants per plate, respectively. Although it is difficult to interpret the frequencies of transfer after overnight growth and mating, approximately 1 in 10^4 of the viable spores generated a neomycin-resistant colony.

Plasmids extracted from two pPM801 and two pPM803 exconjugants were purified and analyzed by restriction enzyme digestion. Analysis of one of these plasmids is shown in Fig. 2. The *Pst*I and *Eco*RI restriction patterns were identical to that of the parental plasmid extracted from donor *E. coli* S17-1. Although contaminating *E. coli* could not be detected, in order to unambiguously confirm the surprising conclusion that these plasmids had indeed been transferred

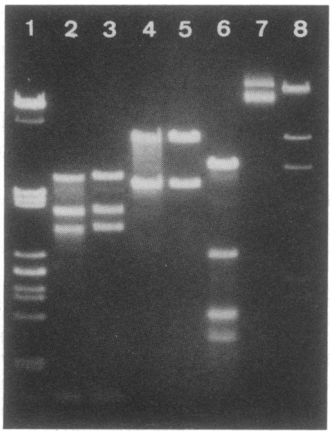

FIG. 2. Agarose gel electrophoresis of pPM801 DNA isolated from *E. coli* (lanes 3, 5, and 7) or *S. lividans* exconjugants (lanes 2, 4, and 6) after digestion with *Pst*I (lanes 2 and 3), *Eco*RI (lanes 4 and 5), or *Bcl*I (lanes 6 and 7). Bacteriophage λ DNA digested with *Pst*I (lane 1) or HindIII (lane 8) was used as molecular size standards.

TABLE 1. Plasmid transfer from *E. coli* to *S. lividans*

Donor strain	No. of exconjugant colonies/plate
294[a]	0
294(pPM801)	0
294(pPM803)	0
S17-1[b]	0
S17-1(pPM801)	2,800
S17-1(pPM803)	1,200
S17-1(pPM804)	0
S17-1(pPM805)	0

[a] *recA* Pro *hsdR hsdM*$^+$.
[b] Strain 294 carrying a chromosomally integrated derivative of RP4.

to *S. lividans*, they were also cut with *Bcl*I. This enzyme cannot cleave DNA isolated from *E. coli*, which is methylated by the *dam* methylase (2); it does cut unmethylated DNA extracted from *S. lividans* (5). The observation that DNA extracted from exconjugants was cut by *Bcl*I proves that the plasmid had indeed been transferred from *E. coli* to *S. lividans*.

The following experiments were done to determine whether plasmid transfer to *S. lividans* was due to a conjugation mechanism or to some hypothetical nonspecific process such as transformation of *S. lividans* by plasmid DNA released from lysed *E. coli*. A series of matings on LB plates supplemented with nalidixic acid (20 μg/ml) was done. Nalidixic acid prevents DNA synthesis in *E. coli*, which is essential for conjugal transfer (12), but does not affect growth of *S. lividans*. No exconjugants were obtained. In addition, neither pPM801 nor pPM803 could be transferred from the parent of S17-1, which lacked *trans*-acting RP4 transfer function (*E. coli* 294) (Table 1). Finally, plasmids lacking *oriT* were nontransferable. In these experiments, plasmids pPM804 and pPM805 were constructed by the procedure shown in Fig. 1, starting from a pAT187 derivative which lacked the *oriT* fragment. These *oriT* mutant analogs of pPM801 or pPM803 could not be mobilized by RP4 *trans*-acting functions (Table 1). The observation that all of these plasmids transformed *S. lividans* with the same efficiency indicated that pPM804 and pPM805 had not sustained unexpected replication defects during their construction.

Matings in liquid were unsuccessful. This might be expected, since IncP1 plasmids conjugate most efficiently on a solid medium (3). Matings on filters applied to agar plates were less convenient and also less efficient by more than 1 order of magnitude. The state of the *S. lividans* recipient was important. Mating with mycelia was not observed, and pregermination of the spores increased the efficiency of transfer by 5 to 10 times.

We tested several other *Streptomyces* spp. as recipients in the same types of crosses. Neomycin-resistant exconjugants were obtained by using *Streptomyces pristinaespiralis* (ATCC 25486) and *Streptomyces viridochromogenes* (DSM 40736). Although repeated attempts were not made, exconjugants were not obtained with *Streptomyces parvullus* (ATCC 12434) or *Streptomyces hygroscopicus* (ATCC 21705).

To our knowledge, this is the first report of the conjugal transfer of a plasmid from *E. coli* to filamentous bacteria of the actinomycete family. In order to accomplish this, we had to supply *oriT* and the complementary transfer functions. The maintenance of the plasmid in the streptomycete recipient was guaranteed by the incorporation of a streptomycete

plasmid. In principle, lysogenic bacteriophages, integrative plasmids, transposons, or homologous recombination could be used to insert transferred DNA into the chromosome. These observations of indiscriminate conjugation have obvious implications with respect to the evolution and exchange of genes in the soil ecosystem. The ability to transfer genetically manipulated DNA sequences from *E. coli* to *Streptomyces* spp. will significantly enhance the possibilities for genetic manipulation in the streptomycetes.

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